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Nucleocytoplasmic Shuttling of Smads 2, 3, and 4 Permits Sensing of TGF-β Receptor Activity

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Summary

Transforming growth factor (TGF)-β stimulation leads to phosphorylation and activation of Smad2 and Smad3, which form complexes with Smad4 that accumulate in the nucleus and regulate transcription of target genes. Here we demonstrate that, following TGF-β stimulation of epithelial cells, receptors remain active for at least 3–4 hr, and continuous receptor activity is required to maintain active Smads in the nucleus and for TGF-β-induced transcription. We show that continuous nucleocytoplasmic shuttling of the Smads during active TGF-β signaling provides the mechanism whereby the intracellular transducers of the signal continuously monitor receptor activity. Our data therefore explain how, at all times, the concentration of active Smads in the nucleus is directly dictated by the levels of activated receptors in the cytoplasm.

Introduction

Upon transforming growth factor (TGF)-β stimulation, the ligand binds the type II receptor (TβRII), which recruits the type I receptor (TβRI/ALK5; hereafter ALK5). TβRII phosphorylates and activates ALK5 which in turn phosphorylates downstream substrates. Several different signaling pathways are now known to operate downstream of TGF-β receptors, but by far the best characterized is the Smad pathway (Massagué et al., 2000; Wakefield and Roberts, 2002). Receptor-regulated Smads (R-Smads), Smad2 and Smad3, are directly phosphorylated and thereby activated by ALK5. Phosphorylation occurs at their extreme C termini on the two most C-terminal serine residues in an "SSXS" motif. Once activated, R-Smads form complexes with the common mediator Smad (co-Smad), Smad4 (Massagué et al., 2000), which are stabilized by the C-terminal phosphoserine residues on the R-Smad (Wu et al., 2001b). The actual stoichiometry of these complexes is not yet resolved; they have been suggested to be either dimers or trimers (Chacko et al., 2001; Wu et al., 2001a, 2001b). The activated Smad complexes accumulate in the nucleus where they are directly involved in the transcriptional regulation of target genes, usually in association with other specific DNA binding transcription factors (Massagué and Wotton, 2000).

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The molecular mechanisms that control Smad subcellular localization and activation are not fully understood. The R-Smads appear to be retained in the cytoplasm in their inactive state, but the mechanism by which this occurs is not resolved (Moustakas et al., 2001). The FYVE domain protein SARA (Smad anchor for receptor activation) that recruits Smads 2 and 3 to the receptor for activation (Tsukazaki et al., 1998) has been suggested to retain the R-Smads in the cytoplasm (Xu et al., 2000). Alternatively, the Smads may be retained in the cytoplasm by their association with microtubules (Dong et al., 2000), and recently, the actin binding protein filamin has been proposed to bind the Smads and to play a role in regulating their activation (Sasaki et al., 2001). It appears that the nuclear localization signal (NLS) in the N-terminal (MH1) domain of the R-Smads is masked prior to activation and that phosphorylation by the receptors results in its exposure (Xiao et al., 2000a, 2000b). In contrast, Smad4 is not actively retained in the cytoplasm in the absence of receptor activation but has been shown to be continuously shuttling between the cytoplasm and the nucleus due to the combined activities of a constitutively active NLS in the MH1 domain and a constitutively active nuclear export signal (NES) in its linker region (Pierreux et al., 2000; Watanabe et al., 2000). The activity of the NES has been shown to depend on the nuclear transport receptor, CRM1 (Pierreux et al., 2000; Watanabe et al., 2000). This is proposed to be masked upon complex formation with activated R-Smads, allowing the complexes to accumulate in the nucleus (Pierreux et al., 2000).

The TGF-β signaling pathway is terminated through receptor degradation, which is mediated by the recruitment of the E3 ubiquitin ligases Smurf1 and/or Smurf2 to the activated receptors by the inhibitory Smad (I-Smad), Smad7 (Ebisawa et al., 2001; Kavsak et al., 2000). This results in the degradation of the receptors and Smad7, and in mink lung epithelial cells (Mv1Lu) the endogenous receptors are substantially degraded 4 hr after TGF-β stimulation (Kavsak et al., 2000). The fate of the activated Smads, however, after prolonged signaling, is not entirely clear. It has been suggested that the activated R-Smads are ubiquitinated and degraded in the nucleus (Lo and Massagué, 1999). However, only a small proportion of the pool of activated R-Smads was demonstrated to be ubiquitinated. When the subcellular localization of the Smads was studied in cells treated with cycloheximide to prevent any new Smad synthesis and then activated for different times by TGF-β, it was clear that the bulk of the nuclear pool of Smad2, Smad3, and Smad4 relocated to the cytoplasm after prolonged TGF-β signaling (Pierreux et al., 2000). Since the R-Smads were dephosphorylated when they appeared in the cytoplasm, it was proposed that they were dephosphorylated in the nucleus by an as yet unidentified phosphatase (Pierreux et al., 2000).

It has long been appreciated that both signal strength and signal duration, dictated by the numbers of receptors activated and the time that they remain active, are important determinants of specificity in terms of biologi-

cal outcome of signaling pathways downstream of growth factor receptors (for reviews see Hill and Treisman, 1995; Marshall, 1995; Schlessinger, 2000). In the TGF-β signaling pathway, the strength and duration of the signal are likely to be reflected by the concentration over time of activated Smads in the nucleus. However, the mechanism whereby the concentration of active Smads in the nucleus directly reflects the activity of the receptors in the cytoplasm over the period of active signaling has not been elucidated. Here we investigate this in detail.

We demonstrate that prolonged activity of the TGF-β receptors (at least 3–4 hr in HaCaT cells) is required for direct TGF-β-responsive transcription and show that continuous TGF-β receptor activity is required to maintain the active Smads in the nucleus. Our data indicate that the Smads are constantly shuttling between the nucleus and the cytoplasm for the duration of active signaling, which provides a mechanism for sensing receptor activity. Active R-Smads in the nucleus are continuously being dephosphorylated which results in their dissociation from Smad4. The Smads recycle back to the cytoplasm independently; Smad4 export from the nucleus is dependent on the nuclear transport receptor CRM1, while Smad2 and 3 are actively exported from the nucleus via a CRM1-independent mechanism. If the receptors are still active in the cytoplasm, the R-Smads are rephosphorylated, form complexes with Smad4, and return to the nucleus. If the receptors are no longer active, the R-Smads are retained in the cytoplasm. These results demonstrate that during active signaling, the Smads constantly monitor the activity of the receptors, which provides a mechanism whereby the levels of active Smads in the nucleus directly reflect the levels of active receptors in the cytoplasm.

Results

The Accumulation of Activated R-Smads in the Nucleus after TGF-β Stimulation Is Slow and Sustained

Upon TGF-β stimulation the activated type I receptor, ALK5, phosphorylates and activates the R-Smads, Smad2 and Smad3. These form complexes with Smad4, which accumulate in the nucleus (Massagué and Wotton, 2000). A time course of accumulation of phosphorylated activated Smad2 (P-Smad2) in the nucleus in the TGF-β responsive human keratinocyte cell line, HaCaT, is shown in Figure 1A (upper panels). It takes approximately 10 min of continuous TGF-β stimulation before nuclear P-Smad2 is detectable. The levels of nuclear P-Smad2 peak at approximately 45 min after TGF-β stimulation and decline after 5 hr but do not decrease to zero even after 8 hr. The accumulation of bulk Smad2 and Smad3 mirrors the behavior of P-Smad2 (Figure 1A, upper panels); levels of PARP act as a loading control. The slow trailing off of the P-Smad2 signal after 5 hr of continuous stimulation is likely due to resynthesis of some component of the pathway (probably both receptors and Smads), since in the presence of the protein synthesis inhibitor, cycloheximide, the disappearance of P-Smad2 from the nucleus occurs more abruptly after 3 hr of continuous TGF-β stimulation (Figure 1A, lower panels; Pierreux et al., 2000). Similar time courses in NIH3T3 fibroblasts revealed largely similar kinetics for accumulation of R-Smads in the nucleus, although the disappearance of the R-Smads from the nucleus after prolonged signaling was slightly faster (by 1 hr; data not shown; Pierreux et al., 2000).

TGF-β Receptors Remain Active for at Least 3–4 Hr Poststimulation

To understand whether the sustained presence of phosphorylated Smad2 (and Smad3) in the nucleus was a consequence of continuous activity of the TGF-β receptors or whether it simply reflected a relatively long half-life of phosphorylated R-Smads, we investigated how long the receptors remain active after TGF-β stimulation.

To do this, we exploited a highly specific and potent ALK5 inhibitor (SB-431542) that we have recently characterized (see supplemental data at http://www.molecule.org/cgi/content/full/10/2/283/DC1; Callahan et al., 2002; Inman et al., 2002; Laping et al., 2002; F.J.N. and C.S.H., submitted). This inhibitor, which acts almost instantaneously, can be used to inhibit ALK5 kinase activity at any point after TGF-β stimulation, and the effects of this receptor inhibition can be compared with the situation in which receptors are allowed to signal continuously. Direct Smad3/Smad4-dependent transcription was investigated using the $CAGA_{12}$-Luciferase reporter gene (Dennler et al., 1998). Direct Smad2/Smad4-dependent transcription was assayed using a DE-Luciferase reporter which is activated by a complex comprising the transcription factor Mixer with activated Smad2 and Smad4 (Germain et al., 2000). TGF-β was added to cells transfected with these reporters and a Mixer expression plasmid where appropriate, and the ALK5 inhibitor was added at different times post-TGF-β addition, with all assays being harvested 8 hr after the TGF-β addition. By determining at what point addition of the ALK5 inhibitor fails to inhibit TGF-β-induced transcription, it is possible to ascertain for how long the receptors remain active and contribute to TGF-β-induced transcription.

In HaCaT cells the receptors remain active for at least 3–4 hr after TGF-β stimulation (Figure 1B). If the receptors are allowed to signal for 3 hr or less, inhibition of TGF-β-induced transcription is observed with both reporters (Figure 1B). However, allowing the receptors to signal for 4 hr before inhibiting them had no effect on TGF-β-induced transcription of the reporters (Figure 1B). Similar experiments were performed in NIH3T3 cells with the only difference being that the $CAGA_{12}$-Luciferase reporter used was stably integrated in these cells. In NIH3T3 cells the TGF-β receptors remained active for at least 2–3 hr (Figure 1C), which is consistent with the observation that the R-Smads remain nuclear for a slightly shorter time in NIH3T3 cells compared with HaCaTs (∼1 hr less; see above).

Taken together, these data indicate that TGF-β receptors remain active for at least 3–4 hr in HaCaT cells and at least 2–3 hr in NIH3T3 cells, and this is required for maximal TGF-β-induced transcription of reporter genes.

To validate this experimental approach and to determine whether the results really reflected the dynamics of receptor activity, we performed an analogous experiment with an unrelated family of receptors that were

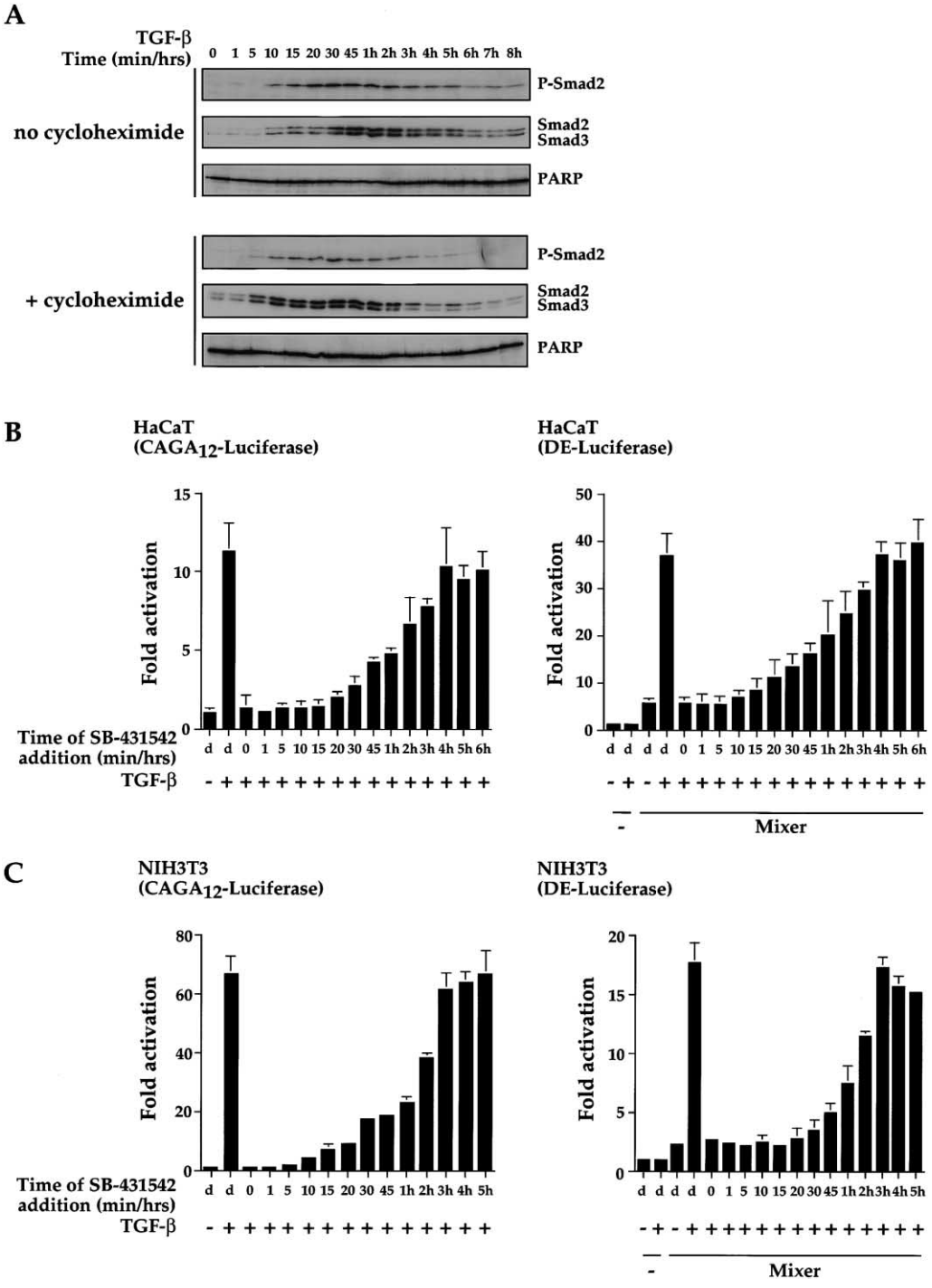

Figure 1. TGF-β Receptors Actively Signal for at Least 3–4 Hr

(A) Time course of Smad phosphorylation and nuclear accumulation in HaCaT cells. Upper panels: HaCaT cells were treated with 2 ng/ml TGF-β1 for the times shown. Nuclear extracts were prepared and analyzed by Western blotting with antibodies against phosphorylated Smad2 (P-Smad2), Smad2 and Smad3, and PARP as a loading control. Lower panels: HaCaT cells were treated as above except that cells were pretreated with cycloheximide (20 μg/ml) for 15 min prior to treatment with TGF-β1.

(B) Continuous ALK5 kinase activity for at least 3–4 hr is required for maximal transcriptional activation of reporter genes in HaCaT cells. HaCaT cells were transfected with either $CAGA_{12}$-Luciferase or DE-Luciferase ± a plasmid expressing Flag-tagged Mixer as indicated. Cells were then treated with or without TGF-β1 for 8 hr in the presence of DMSO (d) or the ALK5 inhibitor, SB-431542 (10 μM), which was added at the times shown after TGF-β1 addition. All samples were harvested together at the 8 hr time point.

(C) Continuous ALK5 kinase activity for at least 2–3 hr is required for maximal transcriptional activation of reporter genes in NIH3T3 cells. An NIH3T3 cell line containing an integrated $CAGA_{12}$-Luciferase reporter (left hand graph) or NIH3T3 cells transfected with DE-Luciferase ± a plasmid expressing Flag-tagged Mixer were treated with TGF-β1 and SB-431542 exactly as in (B). In (B) and (C), the data are means and standard deviations of a representative experiment performed in triplicate.

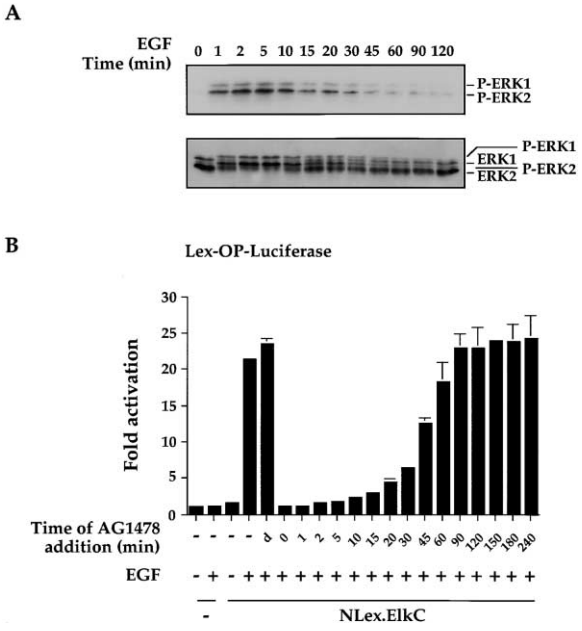

Figure 2. EGF Receptors Actively Signal to ERK1/2 for Only 60–90 Min

(A) Time course of EGF-induced ERK1/2 activation in HeLa cells. HeLa cells were treated with 30 ng/ml EGF for the times shown. Whole-cell extracts were prepared and analyzed by Western blotting with antibodies against phosphorylated ERK1/2 (P-ERK1 and P-ERK2; upper panel), and pan ERK as a loading control (lower panel). Note that the phosphorylated forms of ERK1 and 2 are readily detected with the pan ERK antibody, migrating more slowly than the unphosphorylated forms.

(B) Continuous EGF receptor activity for 60–90 min is required for maximal transcriptional activation of an activated ERK1/2-dependent reporter gene. HeLa cells were transfected with Lex-OP-Luciferase and a plasmid expressing NLex.ElkC as indicated. Cells were then treated with or without EGF for 8 hr in the presence of DMSO (d) or the EGF receptor inhibitor, AG1478, which was added at the times shown after EGF addition. All samples were harvested together at the 8 hr time point. The data are means and standard deviations of a representative experiment performed in triplicate.

known to signal in a transient fashion. We chose the EGF receptor, which is very rapidly activated upon ligand stimulation and then internalized and subsequently targeted for lysosomal degradation (Levkowitz et al., 1998; Schlessinger, 2000; Sorkin, 1998; Waterman et al., 2002). The experiment was performed in HeLa cells which respond well to EGF, and activation of the Ras-ERK MAP kinase pathway was used as a functional readout for the EGF receptors (Yarden and Sliwkowski, 2001). As a transcriptional readout we used a transcription factor fusion protein comprising the C-terminal domain of the Ets transcription factor Elk1 with the bacterial repressor protein LexA (NLex.ElkC), which activates transcription from a Lex operator-driven reporter gene when it is phosphorylated by ERK1 and ERK2 (Marais et al., 1993).

The very rapid activation of the EGF receptors is obvious from the detection of substantial levels of phosphorylated activated ERK1 and ERK2 1 min after EGF stimulation (Figure 2A). The levels of phosphorylated ERK1 and 2 peaked at 5 min, decreased substantially after 30 min, and were virtually back to basal levels at 120 min post-EGF stimulation (Figure 2A). From the transcriptional analysis it was clear that EGF receptors are actively signaling to ERK1/2 for approximately 60–90 min post-EGF stimulation (Figure 2B). Inhibition of the EGF receptors with the specific tyrphostin AG1478 (Levitzki and Gazit, 1995) 60 min or less after EGF stimulation led to an inhibition of EGF-induced transcription. However, inhibition of the receptors after 90 min had no effect on the levels of EGF-induced transcription.

This experiment validates our approach in that transient signaling is readily detected using this experimental design. We therefore conclude that in contrast to tyrosine kinase receptors such as the EGF receptor, signaling from the serine/threonine kinase TGF-β receptors is very prolonged when receptor activity is monitored.

Continuous Recycling of Smads 2, 3, and 4 between the Cytoplasm and Nucleus after TGF-β Stimulation Is Required to Maintain Active Smad Complexes in the Nucleus

We have shown above that continuous receptor activity for at least 3–4 hr in HaCaT cells is required for maximal TGF-β-induced transcriptional activation. However, treatment of these cells with TGF-β leads to complete accumulation of Smad2 and 3 in the nucleus in 30 min as determined by indirect immunostaining (Figure 3A). Nuclear Smad2/3 levels then appear to remain constant for at least 3 hr when assayed in the presence of the protein synthesis inhibitor cycloheximide (Figures 3A and 3B; Pierreux et al., 2000). How, therefore, do the active receptors influence the Smads in the nucleus in the period between 30 min and 3 hr post-TGF-β stimulation?

We speculated that the most likely mechanism involved the Smads cycling between the nucleus and the cytoplasm during this period and tested this directly. HaCaT cells were first pretreated with cycloheximide to prevent any new protein synthesis that could interfere with the interpretation of the experiment (see the Experimental Procedures). Cells were then treated with TGF-β for 1 hr, which is sufficient time for Smads 2, 3, and 4 to accumulate in the nucleus (Figure 3B). At this time, cells were either left or treated with SB-431542 to completely inhibit the ALK5 receptor kinase activity. Cells were fixed at different times, and Smad2/3 and Smad4 were visualized by indirect immunofluorescence (Figure 3B).

In unstimulated cells, Smad2 and 3 are predominantly cytoplasmic, whereas Smad4 is localized throughout the cytoplasm and nucleus as a result of its shuttling between these two compartments (Figure 3B; Pierreux et al., 2000; Watanabe et al., 2000). In cells treated with TGF-β continuously, Smad2, 3, and 4 remained nuclear throughout the time course (Figure 3B). In striking contrast, if ALK5 activity was inhibited at the 60 min time point by addition of SB-431542, the Smads became localized to the cytoplasm at later time points. One hour after the addition of SB-431542 (120 min time point), Smad2 and Smad3 were distributed between the cytoplasm and nucleus, and after a further hour (180 min time point) they were found to be predominantly cytoplasmic and resembled the unstimulated state (Figure 3B, upper panels). Similar behavior was seen for Smad4, although

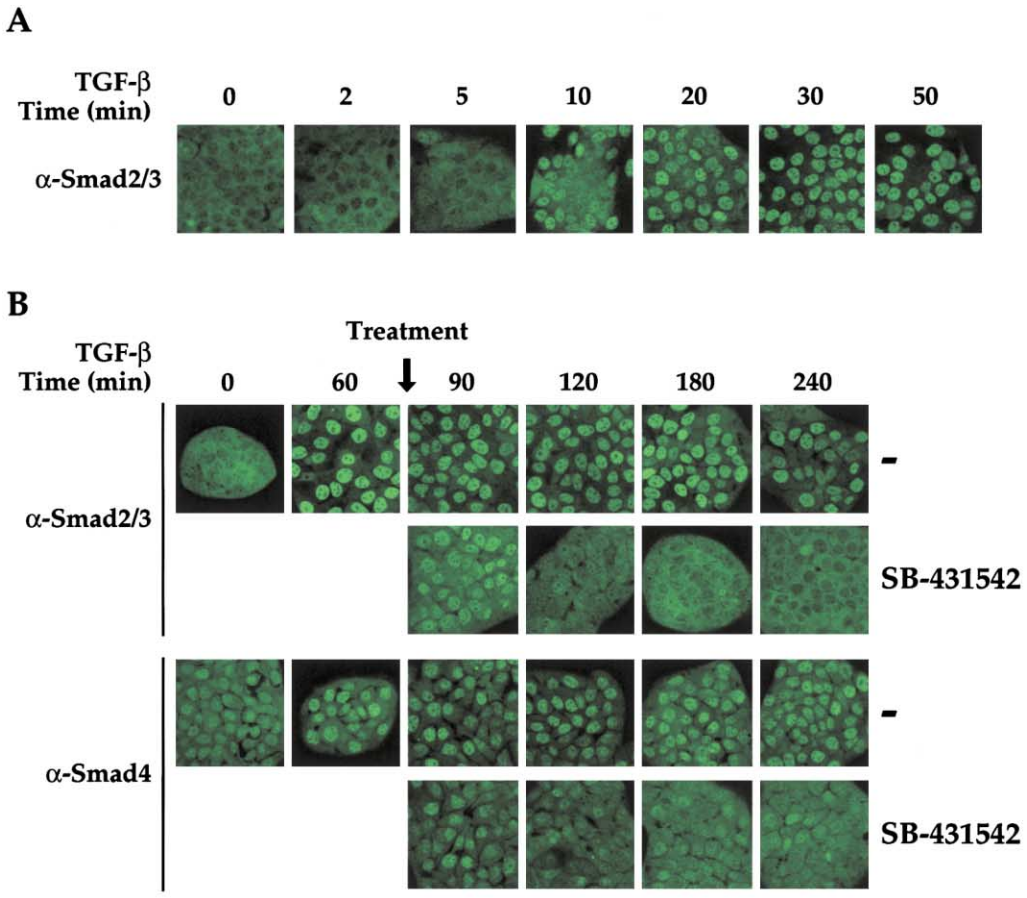

Figure 3. Continuous Recycling of Smads 2, 3, and 4 between the Cytoplasm and Nucleus after TGF-β Stimulation Is Required to Maintain Active Smad Complexes in the Nucleus

(A) Time course of accumulation of Smad2 and Smad3 in the nucleus of HaCaT cells after TGF-β stimulation. HaCaT cells were induced with 2 ng/ml TGF-β1 for the times indicated, and Smad2 and Smad3 were visualized by indirect immunofluorescence using a Zeiss confocal LSM 510 microscope.

(B) HaCaT cells were treated with cycloheximide (20 μg/ml) for 30 min, then induced with 2 ng/ml TGF-β1. Sixty minutes after TGF-β1 addition, cells were either left (no treatment) or incubated with the ALK5 inhibitor, SB-431542 (10 μM), and fixed at the times shown. Samples were processed for indirect immunofluorescence as above using an anti-Smad2/Smad3 antibody or an anti-Smad4 antibody.

(C) Smad2 becomes rapidly dephosphorylated after inhibition of the ALK5 kinase activity. Whole-cell extracts were prepared from HaCaT cells treated identically to those in (B) and analyzed by Western blotting with antibodies against phosphorylated Smad2 (P-Smad2) or Smad2 and Smad3.

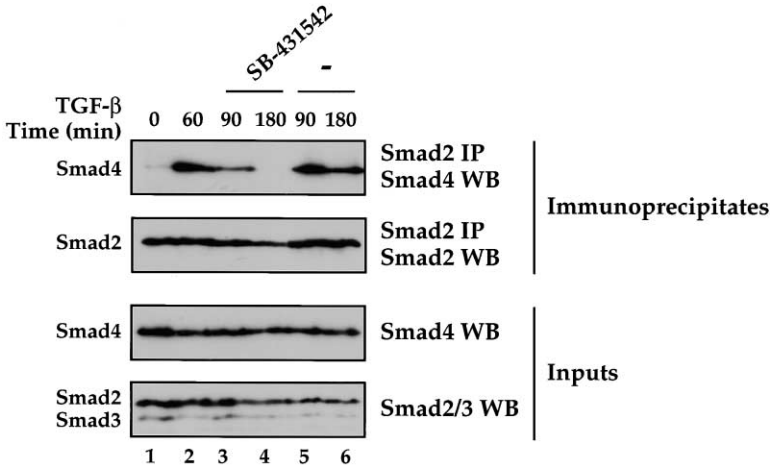

Figure 4. Dephosphorylation of Smad2 after Inhibition of the ALK5 Kinase Activity Leads to Dissociation of Smad2/Smad4 Complexes

HaCaT cells were treated with cycloheximide (20 μg/ml) for 30 min, then induced with 2 ng/ml TGF-β1 for 60 min, at which point cells were either left or treated with SB-431542 (10 μM) for a further 30 min (90 min time point) or 120 min (180 min time point). Whole-cell extracts were immunoprecipitated with a polyclonal anti-Smad2 antibody, then Western blotted with a monoclonal antibody against Smad4 or Smad2/3 (upper panels; Immunoprecipitates). As a control, whole-cell extracts were also Western blotted with the Smad4 or Smad2/3 antibodies (lower panels; Inputs).

cytoplasmic accumulation was never complete, reflecting the unstimulated state (Figure 3B, lower panels).

This experiment reveals that continuous receptor activity is required to maintain active Smads in the nucleus. It indicates that, although the Smads appear to remain statically in the nucleus after TGF-β signaling, they are actually cycling between the cytoplasm and the nucleus.

Once the TGF-β Receptors Are Inactivated, Smad2 Becomes Rapidly Dephosphorylated and Dissociates from Smad4

Active accumulation of the R-Smads and co-Smads in the nucleus after TGF-β signaling requires the formation of Smad2/Smad4 and Smad3/Smad4 complexes (Massagué and Wotton, 2000; Moustakas et al., 2001), which is driven by the receptor-induced phosphorylation of the R-Smad (Chacko et al., 2001; Wu et al., 2001b). The mechanism underlying the nuclear accumulation of the Smad complexes is thought to involve unmasking of NLSs in the Smads, as well as a possible masking of the NES in Smad4 upon complex formation (Pierreux et al., 2000; Watanabe et al., 2000; Xiao et al., 2000a; Xu et al., 2000). We therefore reasoned that, in order for the Smads to exit the nucleus, the R-Smads must be dephosphorylated in the nucleus and the complexes must dissociate.

Western blots were performed on extracts treated identically to those shown in Figure 3B to visualize phosphorylated Smad2, and bulk Smad2 and Smad3. In the absence of SB-431542, the Smad2 remained phosphorylated throughout the time course (Figure 3C, upper panels). However, when SB-431542 was added 60 min post-TGF-β induction, Smad2 rapidly became dephosphorylated (Figure 3C, lower panels). In fact, 60 min of SB-431542 treatment (120 min time point) was sufficient to abolish all Smad2 phosphorylation. There was no degradation of bulk Smad2 and Smad3 during this time course.

We then directly tested by coimmunoprecipitation whether the dephosphorylation of Smad2 led to dissociation of Smad2/Smad4 complexes (Figure 4). HaCaT cells were either treated or not with TGF-β for 60 min, at which point they were either left or treated with SB-431542 for a further 30 min (90 min time point) or 120 min (180 min time point). Complexes of Smad2 and Smad4 were readily detected after 60 min of TGF-β treatment (lane 2). When the cells were treated with SB-431542 for a further 30 min, only low amounts of Smad2/Smad4 complexes were detected (lane 3), when compared with the equivalent sample from cells not treated with SB-431542 (lane 5). When the SB-431542 treatment was for 120 min, which is sufficient to cause complete dephosphorylation of Smad2, no Smad2/Smad4 complexes were detected (lane 4).

Taken together with the data in the previous section, we interpret these results to indicate that under conditions of continuous receptor activation, the R-Smads in the nucleus are continuously being dephosphorylated. They dissociate from Smad4, cycle back to the cytoplasm where they are rephosphorylated by the active receptors, form complexes with Smad4, and return to the nucleus. This activity is revealed when the ALK5 receptor is inhibited by SB-431542, since the Smads that recycle back to the cytoplasm in these conditions are not rephosphorylated by the receptor and thus accumulate in the cytoplasm. This cytoplasmic accumulation takes place with slower kinetics than the ligand-mediated nuclear translocation, suggesting that Smad2/3 import as a complex with Smad4 is faster than Smad2/3 export.

Exit of Smad2 and Smad3 from the Nucleus Does Not Depend on the Presence of Smad4

Since it has been demonstrated that Smad4 continuously shuttles between the cytoplasm and the nucleus (Pierreux et al., 2000), we investigated whether Smad4 was required for the exit of Smad2/3 from the nucleus after receptor inhibition and used the Smad4 null pancreatic cell line, BxPC3, for this experiment (F.J.N. and C.S.H., submitted; Hahn et al., 1996). The design of the experiment was identical to the one shown in Figure 3B. In BxPC3 cells Smad2 and 3 accumulated in the nucleus upon TGF-β stimulation and remained nuclear when TGF-β signaling was continuous (Figure 5A). However, if the ALK5 receptor activity was inhibited by SB-431542, Smad2 and 3 accumulated in the cytoplasm. As with HaCaT cells, this correlated with the dephosphorylation of Smad2 (Figure 5B). Thus, dephosphorylation of the R-Smads and their exit from the nucleus upon receptor inhibition is independent of Smad4.

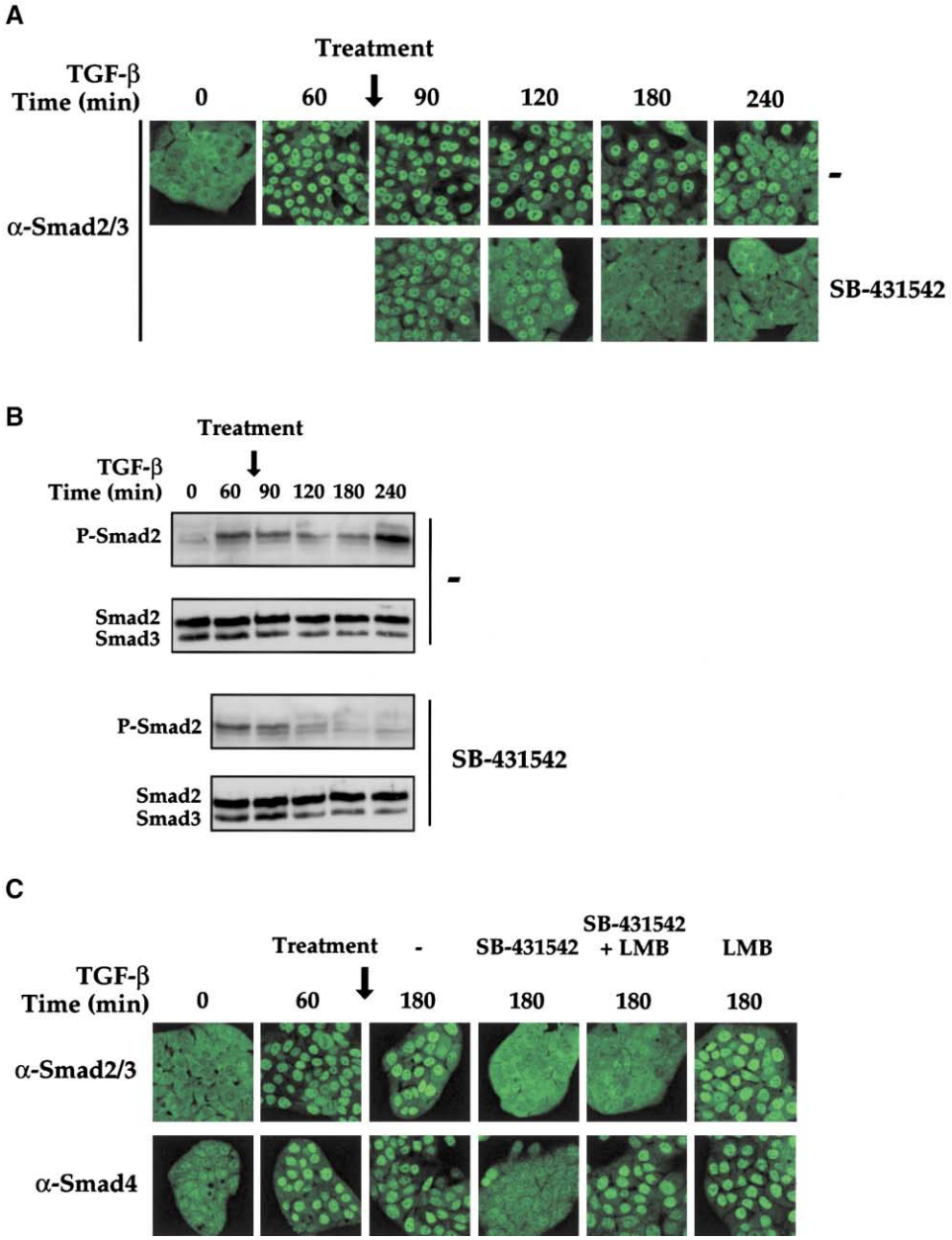

Figure 5. Exit of Smad2 and Smad3 from the Nucleus Does Not Depend on the Presence of Smad4 or on CRM1

(A) Smad4 null cells BxPC3 were treated with cycloheximide (20 μg/ml) for 30 min and then induced with 2 ng/ml TGF-β1. Sixty minutes after TGF-β1 addition, cells were either left (no treatment) or incubated with SB-431542 (10 μM) and harvested at the times shown. Samples were processed for indirect immunofluorescence using an anti-Smad2/Smad3 antibody as above.

(B) Whole-cell extracts were prepared from BxPC3 cells treated identically to those in (A) and analyzed by Western blotting with antibodies against phosphorylated Smad2 (P-Smad2) or Smad2 and Smad3.

(C) Exit of Smad2/3 from the nucleus is independent of CRM1. HaCaT cells were treated with cycloheximide (20 μg/ml) for 30 min and then induced with TGF-β1 for 60 min, at which point cells were either left or treated with SB-431542 (10 μM), LMB (20 ng/ml), or a combination of the two for a further 2 hr (180 min time points). Samples were processed for indirect immunofluorescence as above using an anti-Smad2/Smad3 or an anti-Smad4 antibody.

Smad4 Exits the Nucleus via a CRM1-Dependent Mechanism, but Exit of Smad2 and 3 from the Nucleus Is Independent of CRM1

We previously demonstrated that in unstimulated cells, Smad4 shuttles between the cytoplasm and the nucleus as a result of a constitutively active NLS in its MH1 domain and a constitutively active NES in its linker region (Pierreux et al., 2000). Activity of the Smad4 NES depends on the nuclear transport receptor CRM1, which can be inhibited by the drug leptomycin B (LMB; Kudo et al., 1998; Nishi et al., 1994; Pierreux et al., 2000). Thus, treatment of cells with LMB causes a rapid accumulation of Smad4 in the nucleus (Pierreux et al., 2000). LMB, however, has no effect on the localization of Smad2 and

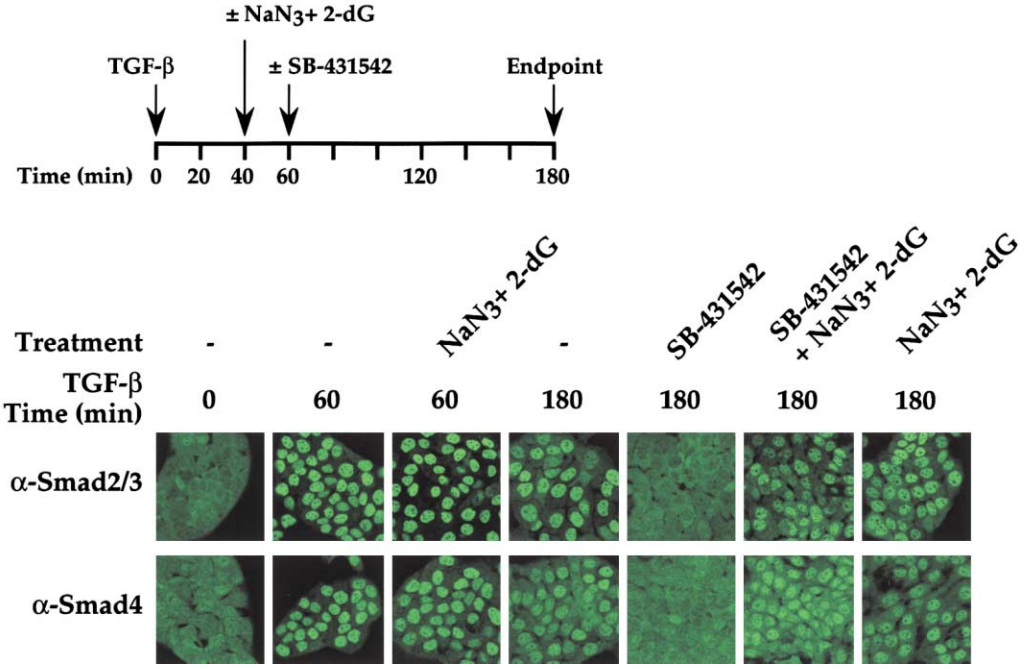

Figure 6. Smad2 and Smad3 Are Actively Exported from the Nucleus

HaCaT cells were treated with cycloheximide (20 μg/ml) for 30 min, and then induced with TGF-β1 for 40 min. At this point, cells were either left or treated with sodium azide (NaN_3) and 2-deoxy-D-glucose (2-dG) in glucose-free DMEM containing 2 ng/ml TGF-β1 and 20 μg/ml cycloheximide to deplete ATP. After 20 min, samples were either fixed (60 min time point), left for a further 2 hr, or treated with SB-431542 (10 μM) for a further 2 hr (180 min time points). Samples were processed for indirect immunofluorescence as above using an anti-Smad2/Smad3 antibody or an anti-Smad4 antibody. A schematic of the experiment is shown above.

3 in unstimulated cells, indicating that in the absence of ligand these Smads are not being exported to the cytoplasm via CRM1 (Pierreux et al., 2000).

We therefore investigated the mechanism by which the Smads exit the nucleus in TGF-β stimulated cells upon premature inhibition of the ALK5 receptor by SB-431542, first testing whether it was CRM1 dependent. The design of the experiment was again as in Figure 3B, except that at the point at which cells were treated or not with SB-431542, LMB was also included in some samples to inhibit CRM1 activity (Figure 5C). Treatment of TGF-β-stimulated cells with SB-431542 led to accumulation of Smad2/3 and Smad4 in the cytoplasm. The addition of LMB inhibited the cytoplasmic accumulation of Smad4 but not of Smad2/3 (Figure 5C).

This result demonstrates that the exit of Smad2/3 from the nucleus is independent of CRM1, while exit of Smad4 occurs via a CRM1-dependent mechanism. Importantly, it also indicates that Smad2/3 exits the nucleus independently of Smad4, which confirms that the complexes must have dissociated in the nucleus.

Smad2 and Smad3 Are Actively Exported from the Nucleus

Exit of Smad2 and 3 from the nucleus is independent of CRM1. The mechanism could involve another nuclear transport receptor (Gorlich and Kutay, 1999; Lipowsky et al., 2000), or, alternatively, they could exit by diffusion, since they are within the size range that can theoretically diffuse through the nuclear pores (Gorlich and Kutay, 1999).

We used ATP depletion to distinguish between these two processes. ATP was efficiently depleted by incubating cells in 10 mM sodium azide and 6 mM 2-deoxy-D-glucose in glucose-free media. This was sufficient to deplete ATP by 67% after 20 min and by 84% after 120 min (see the Experimental Procedures). HaCaT cells were pretreated with cycloheximide and then treated with TGF-β for 40 min. At this point, cells were either left or incubated in sodium azide/2-deoxy-D-glucose in glucose-free media containing TGF-β and cycloheximide. Twenty minutes later, samples for the 60 min time point were fixed, while other cells were treated or not with the ALK5 inhibitor, SB-431542, for 2 hr (180 min time point). Smad2, 3, and 4 were visualized by indirect immunofluorescence (Figure 6). ATP depletion was expected to inhibit the exit of Smad4 from the nucleus after SB-431542 treatment since this is an active CRM1-dependent process. This is clearly demonstrated (Figure 6). In addition, ATP depletion also strongly inhibited the ability of Smad2 and 3 to exit from the nucleus after SB-431542 treatment. Thus, we can conclude that this too is an energy-dependent mechanism and does not occur by diffusion (Figure 6).

Discussion

A Mechanism Whereby the Nuclear Pool of Active Smads Monitors the Levels of Active Receptors in the Cytoplasm

The specificity of biological responses to particular signal transduction pathways can be determined by both

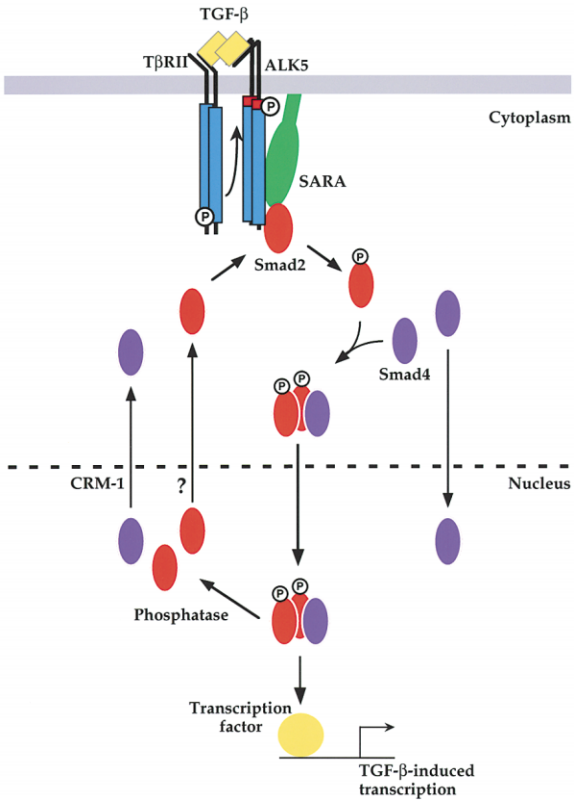

Figure 7. A Model Indicating that Continuous Recycling of Smads between the Cytoplasm and Nucleus Allows Constant Monitoring of Receptor Activity, which Is Required for TGF-β-Induced Transcription

The Smads are depicted as ovals; Smad2 is orange and Smad4 is purple. A Smad-recruiting transcription factor is depicted as a yellow circle. Only the behavior of one of the R-Smads, Smad2, is shown for clarity. For details see text.

the strength and duration of the signal (for reviews see Hill and Treisman, 1995; Marshall, 1995; Schlessinger, 2000). Thus, mechanisms must exist for the nuclear end of the signaling pathway to sense changing signal intensities which are reflected in the level of activated receptors at the plasma membrane (Freeman and Yamamoto, 2001). In addition, the pathway must be able to terminate efficiently and be able to rapidly reinitiate.

Our observation that, after TGF-β stimulation, the TGF-β receptors remain active for at least 3–4 hr in the epithelial cell line, HaCaT, and that the activity of the receptors during that time period is required for maximal TGF-β-induced transcriptional activation led us to investigate how the activity of receptors in the cytoplasm is sensed by the Smads in the nucleus. Our data suggest a simple mechanism whereby the nuclear Smads are in constant communication with the cytoplasm, monitoring the levels of activated receptors. It explains how the levels of nuclear Smads will directly reflect the activity of the receptors at all times.

A model is shown in Figure 7 that depicts this dynamic signaling process. The essence of the model is that rather than existing as a static pool of activated Smads in the nucleus, the R-Smads are being constantly dephosphorylated, which results in dissociation of the R-Smad/co-Smad complexes and export of the inactive Smads to the cytoplasm. If the receptors are still active, the Smads will be reactivated and return to the nucleus. If the receptors are no longer active, the Smads accumulate back in the cytoplasm. We conclude that the complexes dissociate in the nucleus because the export of Smad2 and 3 from the nucleus occurs independently of Smad4 export. Smad4 export depends on the nuclear transport receptor, CRM1, while Smad2 and 3 are actively exported via a CRM1-independent mechanism. Since recent work has demonstrated that the complex formation is mediated by the phosphoserine residues on the R-Smads (Wu et al., 2001b), we conclude that dephosphorylation of the R-Smads by an as yet unidentified nuclear phosphatase is responsible for triggering complex dissociation.

The model provides an explanation for the continuous shuttling activity of Smad4. It has recently been proposed that the mechanism for ensuring that R-Smad/co-Smad heteromeric complexes form rather than homomeric complexes depends on the presence of Smad4 in the vicinity of the activated receptor (Wu et al., 2001b). Given that Smad4 is not in large excess over Smad2 and 3 (our unpublished data), it is important for Smad4 to exit the nucleus at the same time as the inactive Smad2 and 3, since it has to be present in the cytoplasm when the R-Smads are rephosphorylated.

Two molecules important for R-Smad recycling remain to be identified: the nuclear phosphatase and the nuclear exporter. Attempts to inhibit the phosphatase activity with a variety of cell-permeable phosphatase inhibitors have not been successful, and thus its identification remains a challenge for the future. Our data indicate that the nuclear transport receptor responsible for exporting inactivated R-Smads from the nucleus is distinct from CRM1. Like CRM1, it is likely to be a member of the importin β superfamily of nuclear transporters, which are characterized by a RanGTP binding motif, and include both the importins and exportins. There are thought to be at least 22 transport receptors in higher eukaryotes (Lipowsky et al., 2000), but apart from CRM1 (for which there is a specific inhibitor) the other exportins remain not well characterized with regard to their cargos.

Another important consideration for our model is the location of the active TGF-β receptors during signaling. Several possibilities exist. The receptors, once activated, may remain at the plasma membrane. They may be internalized via the endocytic pathway or, alternatively, shuttle between these two compartments as demonstrated for the EGF receptors (Levkowitz et al., 1998). TGF-β receptors have been shown to internalize, although it is not clear whether this is required for signaling (Moustakas et al., 2001). It will be important to determine the location of the receptors during the course of signaling, since this has obvious implications for the sensitivity of the system to fluctuations in external ligand concentration.

A previous model for TGF-β signaling suggested that termination of the signaling pathway occurred through the ubiquitination and degradation of the nuclear phosphorylated R-Smads (Lo and Massagué, 1999), implying that signaling to the nucleus by the Smads was a one-way process. In contrast, our data demonstrate that

the Smads continuously cycle between the nucleus and cytoplasm, enabling them to sense the duration of receptor activity. These apparent contradictions, however, may be reconciled. First, the view that the phosphorylated nuclear Smad2 was degraded and not dephosphorylated relied on the use of proteasome inhibitors, which will stabilize any cellular components that are normally degraded via the proteasome. This will include the activated TGF-β receptors, which are now known to be degraded after activation (Ebisawa et al., 2001; Kavsak et al., 2000). It is clear from our present data that stabilizing the receptors will result in an extension of the lifetime of phosphorylated Smad2 in the nucleus, apparently stabilizing it. Second, whereas our experiments examine the bulk pool of Smads, only a very small proportion of the pool of nuclear Smads was demonstrated to be ubiquitinated and targeted for degradation (Lo and Massagué, 1999). One possibility is that this is the small pool of R-Smad that targets the corepressor SnoN for degradation (for a review see Moustakas et al., 2001). Another (not mutually exclusive) possibility is that the activated R-Smads that are ubiquitinated are those actively engaged in activated transcription (Fukuchi et al., 2001). Indeed, recent work has suggested that ubiquitination of transcriptional activation domains of transcription factors serves as a dual signal for activation and subsequent activator destruction (Salghetti et al., 2001).

Signal Duration Is a Determinant of the Specificity of the Biological Responses to TGF-β

In a separate study (F.J.N. and C.S.H., submitted), we have demonstrated that the duration of TGF-β signaling, measured by the time of residence of active Smad complexes in the nucleus, is an important determinant of specificity of the biological response. We have shown that pancreatic tumor cell lines that contain low levels of type I TGF-β receptors exhibit a very transient accumulation of active Smads in the nucleus compared with a pancreatic tumor cell line containing higher levels of type I receptors, which shows a prolonged accumulation of the active Smads in the nucleus. Strikingly, we found that the cells with the transient signaling pathway have specifically lost their ability to growth arrest in response to TGF-β, while prolonged signaling is associated with TGF-β-induced growth arrest. It is now known that the activated receptors are degraded via a mechanism involving Smad7 and the E3 ubiquitin ligases, Smurf1 and/or Smurf2. If the rate of receptor degradation is constant, receptor levels would fall below a threshold for signaling more rapidly if the receptors are present at a lower level than at a higher level. Now that we have shown that the Smads are constantly recycling between the cytoplasm and nucleus, we can readily explain how the levels of receptor could directly determine how long a pool of activated Smads remains in the nucleus, which in turn could influence the biological response to TGF-β.

We have elucidated a mechanism whereby the nuclear components of a signaling pathway continuously sense the levels of activated receptors in the cytoplasm. Signaling pathways that transduce signals from activated receptors at the plasma membrane to the nucleus to influence transcriptional activation all require a molecule to be transported from the cytoplasm to the nucleus. These may be transcriptional regulators such as the Smads, STATs, or β-catenin, or kinases such as ERK MAP kinase. It will be very interesting to determine whether constant recycling of components between the nucleus and cytoplasm during the period of active signaling is a feature of other signaling pathways.

Experimental Procedures

Plasmids

The following plasmids have previously been described: mammalian expression plasmid encoding NLex.ElkC (Marais et al., 1993), EF-Flag-Mixer (Germain et al., 2000), EF-LacZ and the DE-driven Luciferase reporter (Pierreux et al., 2000), $CAGA_{12}$-Luciferase reporter (Dennler et al., 1998), Lex-Op-Luciferase reporter (Gineitis and Treisman, 2001), and pTK-Neo (Cruzalegui et al., 1999).

Cell Culture, Transfections, Inductions, and Inhibitors

The NIH3T3-$CAGA_{12}$-Luciferase cell line was generated by cotransfecting $CAGA_{12}$-Luciferase with pTK-Neo and selecting clones in media containing 1 mg/ml G418 (Life Technologies). Stable clones were then maintained in DMEM/10% FCS/0.4 mg/ml G418. HaCaT, NIH3T3, and HeLa cells were all maintained in DMEM/10% FCS, and the Smad4 null cell line, BxPC3 was maintained in RPMI/10% FCS. NIH3T3 cells and HeLa cells were transfected using LipofectAMINE (Life Technologies), and HaCaT cells were transfected using DEAE-dextran (Hill et al., 1993).

TGF-β1 (PeproTech) was dissolved in 4 mM HCl/1 mg/ml BSA and used at a final concentration of 2 ng/ml. SB-431542 was dissolved in DMSO and used at a final concentration of 10 μM throughout. Where cycloheximide was used, it was added at 20 μg/ml 30 min prior to treatment with TGF-β1 (Figures 3, 4, 5, and 6) or 15 min prior to TGF-β1 (Figure 1). We have demonstrated that this concentration of cycloheximide is sufficient to inhibit protein synthesis by 93% (Pierreux et al., 2000). Leptomycin B (LMB) was resuspended at a concentration of 10 μg/ml in ethanol and used at a final concentration of 20 ng/ml. To deplete cells of ATP, cells were treated with sodium azide (10 mM) and 2-deoxy-D-glucose (6 mM) in glucose-free DMEM 20 min before treatment with SB-431542. EGF (R&D Systems) was dissolved in 10 mM acetic acid/0.1% BSA and used at 30 ng/ml. The EGF receptor inhibitor, AG1478 (Calbiochem), was dissolved in DMSO and used at a final concentration of 3 μM. Cellular ATP concentrations were quantitated using the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's instructions.

Cell Extracts and Transcriptional Assays

Nuclear extracts were prepared as described (Wong et al., 1999). Whole-cell extracts were prepared as described (Inman et al., 2002). Transcription assays were as described (Pierreux et al., 2000).

Immunofluorescence, Western Blotting, and Immunoprecipitations

Indirect immunofluorescence microscopy was performed exactly as described (Pierreux et al., 2000) except that after the final washing steps, the cells were refixed with 4% formaldehyde in PBS for 10 min prior to mounting in Vectashield. Samples were analyzed using a Zeiss confocal LSM 510 microscope.

Western blotting was performed using standard techniques. The following antibodies were used for Western blotting and/or immunofluorescence: monoclonal antibody against Smad2 (which also recognizes Smad3; Transduction Laboratories); monoclonal antibody against Smad4 (B8; Santa Cruz); polyclonal antibody against poly (ADP-ribose)-polymerase (PARP; Roche); polyclonal antibody against phosphorylated activated Smad2 (Faure et al., 2000); polyclonal antibody against pan ERK, and a monoclonal antibody against phosphorylated ERK1 and ERK2 (NEB).

Immunoprecipitations (IP) followed by Western blotting were performed essentially as described (Pierreux et al., 2000). The polyclonal antibody against Smad2 used for the IP was previously described (Nakao et al., 1997), and IPs were blotted with the

monoclonal antibodies against Smad4 and Smad2/3 as described above.

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